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Growth Performance of *Bacillus amyloliquefaciens* (Fukumoto 1943) in Different Fermentation Processes

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Abstract: *The potential of microorganisms as biotechnological sources of industrially pertinent product has stimulated interest in the exploration of microbes having diverse activity. This research has been carried out to explore the potentiality of an important bacterium, Bacillus amyloliquefaciens to grow in different types of fermentations i.e. submerged fermentation, solid state fermentation (SSF), biofilm fermentation and comparative observation of solid and liquid fermentation by cell counting at different time duration. In this context, submerged fermentation was simply carried out with cultured strain in LB media. In solid state fermentation "Okara" (soy-residue) was used as substrate and found to be the best when moistened with glucose solution and in biofilm fermentation, production media and modified production media were used for the formation of film on the surface of the beaker. Result revealed that biofilm formation was facilitated by modified LB media. Since this strain have broad substrate spectrum for growth and different conditions may be needed for scale up process, so further research is required to establish suitable condition in laboratory as well as industry scale. Besides, molecular biology approaches can help to modify the strain to overcome different difficulties and to enhance gross production of the desired products.*

Keywords: *Growth performance, Bacillus amyloliquefaciens, submerged fermentation, Solid state fermentation, Biofilm formation*

I. Introduction

Bacillus amyloliquefaciens (Fukumoto 1943) is a non-pathogenic, rod shaped and catalase positive bacteria (Loncar et al., 2014). It is a spore forming gram positive bacterium and its spore survive more than 80°C (Valerio et al., 2012).

Like other bacteria from genus *Bacillus*, *Bacillus amyloliquefaciens* is used for probiotics production in different sectors like poultry farm, aquaculture etc. (Hong et al., 2005). This bacterium is not only used for probiotic but it has diverse role in enzyme production. The production of amylases is overshadowing all other enzymes; hence, amylases account 65% of enzyme market in world (Van Der Maarel et al., 2002; Balkan and Ertan, 2007). Alpha amylase is currently being produced by this bacterium using different substrates (Abd-Elhalem et al., 2015).

Now a days *Bacillus amyloliquefaciens* is widely used for the production of poly glutamic acid (PGA) (Feng et al., 2015). PGA is a biodegradable anionic polymer that is water soluble and non-toxic to human and environment (Goto and Kunioka, 1992). It is mainly used in cosmetics industries, food industries, construction industries, agriculture sphere, medical sphere, sanitary sphere etc. In the present world, consumers are more aware of the adverse effects of chemical preservatives and prefer foods containing natural and safe preservatives (Settanni and Corsetti, 2008) is desirable if generally recognized as safe (GRAS) organisms or metabolites from GRAS organisms that effectively inhibit fungi are used as preservatives against fungal infection. In this respect, *Bacillus amyloliquefaciens* and its metabolites have great potential. Bacilli, natural soil residents, have been known to produce more than two dozen different antimicrobial compounds and, thus, effectively compete with other microorganisms, such as fungi, in the same environment (Stein, 2005). *Bacillus amyloliquefaciens* produces volatile compounds (VOCs) that inhibit the growth and spore germination of fungus like *Fusarium oxysporum* f. sp. *Cubense (*Yuan et al.,2012).

For diverse applications of *Bacillus amyloliquefaciens*, it is very important to know the growth condition of this bacterium. In this experiment, we isolated *Bacillus amyloliquefaciens* strains and tested its growth performance in different conditions e.g. submerged fermentation, solid state fermentation, biofilm fermentation and comparative analysis among these fermentations in the perspective of Bangladesh.Therefore,

this study has carried out to know the growth performance of *Bacillus amyloliquefaciens* in different fermentation conditions along to check the formation of biofilm.

II. Materials and Methods

2.1 Study Location

The study was carried out in the laboratory of Department of Biotechnology and Genetic Engineering, Jahangirnagar University, Savar, Dhaka.

2.2 Isolation of spore forming bacterium *Bacillus amyloliquefaciens* from a probiotic

In this study *Bacillus amyloliquefaciens* RB-14 strain was isolated from an eatable probiotic by the method previously described (Barbosa et al., 2005) with slight modifications. Briefly, the sample was initially mixed with buffered peptone-water in 1:1 ratio before thoroughly mixing. The sample was 10-fold diluted and incubated for 24 hours (hrs). After incubation, the cultures were heated shock at 80°C for 10 minutes in order to select against vegetative cells. Subsequent isolation was carried out by streaking heat treated cultures on isolation media. The uniformity of the bacterial colony was checked on the agar plate and the strain was kept at -5°C.

2.3 Submerged fermentation with *Bacillus amyloliquefaciens*

Isolated *Bacillus amyloliquefaciens* RB-14 strain was used for the fermentation. The strain was cultured in the LB media. Sterilization of the media and all other necessary materials were done by autoclaving at 121°C and 15 lbs for 20 minutes and then inoculated with the freeze stored strain after thawing for 5 minutes at room temperature. Five test tubes were taken for the culture of the bacteria. In each test tube 5ml autoclaved media and 100 microlitre stock cultures of *Bacillus amyloliquefaciens* was added into each test tube. The total process of inoculation was completed in the laminar air flow.

Test tubes were kept at the shaking water bath for culture for 72 hours. Cells were counted at 0hrs, 3hrs, 9hrs, 24hrs, and 48 hrs and at 72 hrs after inoculation. For cell counting agar plates were prepared adding 2% agar into the LB media. Serial dilution process was observed for cell counting.

In serial dilution 1ml fermented culture and 9ml sterile NaCl were mixed in each test tube. 100 microlitre cultured bacteria from diluted test tubes were taken for plating onto the agar media. They were cultured on the plate for 18 hours and then the colonies were counted. pH was measured at 0 hr, 3 hrs, 9 hrs, 24 hrs, 48 hrs (at the same hours when cells were counted).

2.4 Solid state fermentation with *Bacillus amyloliquefaciens*

Okara (soy-residue), a cellulosic by product of the soymilk and tofu (soy paneer) in industries was used as the substrate for solid state fermentation (Khare et al., 1995), when moisture free, okara contains 8 to 15% fats, 12 to 14.5% crude fiber and 24% protein, and contains 17% of the protein from the source soybeans.

Total fermentation procedure was divided in two steps. Firstly, okara fermentation with 75% moisture (okara 37.5 g; water 112.5 ml) and then okara fermentation with glucose (total 5% glucose, 2.5 g glucose in 20ml solution). For the former step, total okara were equally distributed in three 500 ml beakers for fermentation (12.5 g okara in each beaker).

In each beaker 15 microlitre culture (23 hrs cultured) of *Bacillus amyloliquefaciens* was added and mixed properly with sterilized spatula. They were kept for fermentation for 72 hrs. Colony Forming Units (CFU) counting was performed by serial dilution (After 72 hrs). For this, 1g fermented okara and 9ml water were mixed.100 microliter culture was poured into each LB plate. In no-7 dilution we counted the cells in the LB plate. We got different cells number from each process.

2.5 Biofilm formation with *Bacillus amyloliquefaciens*

2.5.1 Media preparation for biofilm fermentation

For Biofilm formation LB medium was used containing peptone (3%), glucose (1%), potassium dihydrogen phosohate (0.1%), epsom salt (0.05%), pH was adjusted to 6.8. The effect of supplementing growth media with varying concentrations of carbon sources on biofilm growth was investigated in this study. The biofilms were grown in the glass beaker and quantified via crystal violet staining. The media used in this study were LB and a modified LB broth (LBM) to which potassium dihydrogen phosphate (KH_2PO_4) was added.

III. Results and Discussion

3.1. Submerged fermentation

In this study, cells were counted by serial dilution from the fermented media. Cells were counted on the agar plates. Operating parameters such as pH, temperature, oxygen consumption, and carbon dioxide formation

are measured and tightly controlled (Pinheiro et al., 2006; Aguilar et al., 2008). Table 1 shows the cell numbers and pH profile at different hours of incubation.

Table 1. Cell number and pH value in submerged fermentation at different times.

Hours	Colony Forming Units (CFU)	pH
0	$1.3x10^6$	6.54
3	$1.6 x10^7$	6.38
9	$2.0x10^9$	6.24
24	$1.0x10^9$	6.61
48	$5.0x10^7$	7.33
72	$1.65x10^7$	7.16

The number of cell is very important for different metabolites production through submerged fermentation. The highest cellular growth was found at 9 hours of incubation then it gradually decreased. Result also demonstrated that pH values of the media decreased initially due to the increased number of cells and their higher catabolic activity. However, gradually at longer incubation medium pH increased to basic level due to ammonification and breakdown of nitrogenous compounds in the medium components (Aguilar et al., 2001; Papagianni et al., 2004; Zohora et al., 2009). The initial increased cell number and medium pH and later decreased medium pH and cellular growth of *Bacillus amyloliquefaciens* for submerged fermentation coincide with the findings of Gibbs et al., 2000 and Velazquez-Cedeno et al., 2002.

3.2. Solid state fermentation (SSF)

Okara (soybean curd residue), rich in water-insoluble ingredients, is a useful substrate for microbial fermentation. Ahamad et al., (2010) successfully produced an antibiotic iturin from okara. In this experiment okara was used as the substrate for SSF. Fermentation process was performed with glucose and moisture. Serial dilution procedure was used for the measurement of the cell numbers. 1g fermented okara and 9ml water was mixed properly for the serial dilution procedure. In step 1, (fermentation with moisture) $3.5x10^9$ cells were counted from 1g fermented okara, so the total number of cells from 12.5g was $4.37x10^{10}$, whereas in step-2 (Fermentation with glucose), from12.5 g of fermented okara $9.0x10^{10}$ cells were calculated (1g fermented okara containing $7.2x10^9$ cells) (Fig. 1). This finding revealed that glucose fermented okara contains two times more cells from than the moisture fermented okara. Solid-state fermentation (SSF) has built up credibility in recent years in biotech industries due to its potential applications in the production of biologically active secondary metabolites, apart from feed, fuel, food, industrial chemicals and pharmaceutical products and has emerged as an attractive alternative to submerged fermentation. Bioremediation, bioleaching, biopulping, biobeneficiation, etc. are the major applications of SSF in bioprocesses which have set another milestone (Singhania et al., 2009).

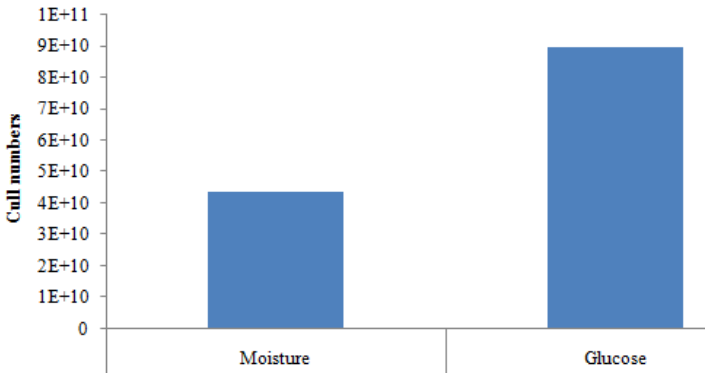

Figure 1. Comparison between moisture and glucose culture on the basis of cell number.

3.3. Biofilm formation

This study was also aimed to check the formation of biofilm with *Bacillus amyloliquefaciens*. We found that by modifying LB by adding potassium dihydrogen phosphate, biofilm growth was facilitated (Fig. 2). Biofilm growth was substantially stimulated over a wide range of concentrations for several of the carbon sources investigated. Furthermore, the distinct adhesion patterns for biofilm growth observed varied according to the media, concentration and carbon source utilized. Aboulkacem et al., 2014 reported that *Bacillus amyloliquefaciens* formed weak biofilm rings at the gas/liquid interface when grown in LB despite supplementation with various carbon sources. Stimulated biofilm growth and two distinct adhesion patterns were observed when cells were grown in supplemented LBM (Jones et al., 2005). However, time course

experiments elucidated an alternative growth pattern. Biofilm staining patterns monitored over hourly time points indicated that under these conditions initial adhesion occurred at the bottom of the tube, and as the biofilm increased in density it spread over the entire surface upward toward the gas/liquid interface (Morikawa et al., 2006). The time course experiments also revealed that the earliest point at which adhesion to the beaker surface could be detected as well as the point of maximum quantifiable biofilm density was dependent on the varying concentrations of the carbon sources present. In the presence of the rapidly metabolizable sugar glucose, biofilm growth was stimulated at concentrations ranging from 0.2% to 7%, with a maximum stimulation of approximately 2.7 fold as compared to the control in the absence of glucose.

Figure 2. Biofilm formation by *B. amyloliquefaciens* in LB media

IV. Conclusion

There is a continuous development in the research field of *B. amyloliquefaciens* over the last couple of years. Our main target was to establish a suitable laboratory condition at which the growth of this bacterium is maximum and also observe the biofilm formation pattern. We found maximum growth in solid state fermentation when moistured with glucose. We also found biofilm formation was facilitated by modified LB media. Since this strain have broad substrate spectrum for growth and different conditions may be needed for scale up process, so further research is required to establish suitable condition in laboratory as well as industry scale. Besides, molecular biology approaches can help to modify the strain to overcome different difficulties and to enhance gross production of the desired products.

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